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13. ABSTRACT (Maximum 200 Words)

Hypoxia-inducible factor-1 (HIF-1) is an important transcriptional factor that is activated in conditions of decreased oxygen. It mediates cell survival in hypoxia by promoting genes involved in glucose homeostasis, erythropoiesis, and angiogenesis. Manganese superoxide dismutase (MnSOD) is an important antioxidant enzymes involved in cancer cell growth. Our group has shown both *in vitro* and *in vivo* that both tumor growth and vascularization are suppressed with increased MnSOD. We hypothesize that the SOD enzymes and HIF-1 are linked and that SOD overexpression in malignant tumors can modulate HIF-1α protein levels.

To test this hypothesis we exposed MnSOD overexpressing cells to hypoxia and examined HIF-1α protein. Our results demonstrated that increase of MnSOD (3-30 fold activity) in breast cancer cells suppressed the accumulation of HIF-1α protein under hypoxia in a dose dependent manner. These results suggest that MnSOD regulates HIF-1α and that the modulation of HIF-1α protein levels may account for the tumor suppressor function of SOD enzyme.

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Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....5

Key Research Accomplishments.....13

Reportable Outcomes.....14

Conclusions.....15

References.....16

Appendices.....

Introduction

When mammalian cells are exposed to the reduced levels of oxygen and nutrients, the growth and viability is reduced [Dunn T, 1997]. Normal tissue displays an oxygen gradient across a distance of 400 μm from a blood supply. On the contrary, tumor cells adjacent to capillaries display a mean oxygen concentration of 2%, and cells located 200 μm from the nearest capillary display a mean oxygen concentration of 0.2% [Shonat RD & Johnson PC, 1997]. Tumor cells live in an acidic environment in contrast to normal tissue because of production of lactate and other acids by metabolism under hypoxic conditions [Wike-Hooley JL *et al.*, 1984]. Therefore, the survival of tumor cells partly depends on the ability to recruit new bloodvessels through angiogenic factors.

Human tumors can survive under extreme hypoxia, which indicates that their ability to adapt to hypoxic conditions plays a critical role in tumor progression. Generally, cells respond to hypoxic conditions by altering patterns of gene expression. Among the first gene products that are stimulated to over-express at the onset of hypoxia is hypoxia-inducible factor-1 (HIF-1) [Wang GL & Semenza GL, 1993b]. Thus, HIF-1 is highlighted on the stage of hypoxia responses. HIF-1 is a transcription factor, which mediates crucial homeostatic responses to reduced O_2 availability in mammals by inducing angiogenesis, glycolysis and erythropoiesis [Carmeliet P *et al.*, 1998].

HIF-1 is a heterodimeric transcription factor that mediates essential homeostatic responses to reduced O_2 availability in mammals. The HIF-1 heterodimer is composed of an HIF-1α subunit and an HIF-1β subunit (also known as aryl hydrocarbon receptor (AHR) nuclear translocator (ARNT)) [Wang GL *et al.*, 1995]. HIF-1α is an 826 amino acid protein, which is unique to HIF-1. It is degraded in normoxic conditions by the ubiquitin-proteosome pathway [Huang LE *et al.*, 1996], but under hypoxic conditions is stabilized and imported into the nucleus [Kallio PJ *et al.*, 1997]. Once there, HIF-1α recruits the CREB-binding protein (CBP/p300 coactivator protein) and then heterodimerizes with ARNT [Kallio PJ *et al.*, 1998].

HIF-1 expression correlates with progression and angiogenesis in tumors [Zagzag D *et al.*, 2000]. In highly vascularized tumors, HIF-1α expression is higher than that in lower vascularized tumors.

Manganese superoxide dismutase (MnSOD) and Copper-Zinc superoxide dismutase (CuZnSOD) have been shown to play an important role in preventing the development of cancers [Leuthauser SW *et al.*, 1981; Oberley LW & Oberley TD, 1988]. MnSOD is located in the mitochondrial matrix whereas CuZnSOD is in the cytosole. They both convert superoxide ($O_2^{\bullet-}$) to produce hydrogen peroxide (H_2O_2) during normal oxygen metabolism and contribute to the redox state of the cell [Wong GH *et al.*, 1989]. While $O_2^{\bullet-}$ is compartmentalized and is thought to remain close to the area of its production, H_2O_2 is highly diffusible. In general, MnSOD activity is low in cancer cells when compared to their normal cell counterparts [Oberley LW & Buettner GR, 1979]. Our group has shown both *in vitro* and *in vivo* that malignant phenotype is suppressed when MnSOD is overexpressed [Li N *et al.*, 1998; Zhang HJ *et al.*, 1999; Zhong W *et al.*, 1999; Li S *et al.*, 2000]. It has been implicated as a tumor suppressor and as a metastasis suppressor in some tumor cell lines.

Body

The objective for this research is to better understand the relationship of MnSOD activity with HIF-1 expression. This is the first time that a connection between MnSOD antioxidant capacity and tumor angiogenic potential has been proposed. If MnSOD prevents hypoxic induction of HIF-1, it will provide an exciting new opportunity for therapeutic intervention. And indeed, MnSOD may offer an opportunity to significantly lower the mortality associated with angiogenesis and metastasis of human cancers.

We **hypothesize** that modulating MnSOD activity levels in malignant breast cancer cells will suppress HIF-1α protein accumulation and thus regulate HIF-1 transcriptional activity.

To test this hypothesis we first transfected human breast carcinoma MCF-7 cells with human MnSOD cDNA to increase the expression of MnSOD in these cells. And then MnSOD overexpressing cells were exposed to hypoxia and HIF-1α protein levels was examined. Our results demonstrated that increase of MnSOD (3-30 fold activity) in breast cancer cells suppressed the accumulation of HIF-1α protein under hypoxia in a dose dependent manner. This is the first time that a direct connection between HIF-1 alpha and SOD has been shown. These results indicate that SOD regulates HIF-1 alpha and the modulation of HIF-1 alpha protein levels may account for the tumor suppressor function of SOD enzymes.

The following tasks outlined in the approved Statement Of Work has been accomplished:

Task 1: Development of breast cancer cells with altered MnSOD expression.

1. We have transfect breast carcinoma MCF-7 cells with MnSOD and vector controls.
2. For stable transfection: human MnSOD cDNA has been inserted into appropriate expression vectors. For transient transduction, adenovirus containing human MnSOD cDNA has been induced into MCF-7 cells.
3. Plasmid transfected cells have been characterized regarding their growth curve, doubling time and antioxidant profile.

Task 2: Determine if level of MnSOD activity alters HIF-1 activation in human breast cells.

1. Cells were subjected to conditions known to activate HIF-1. (hypoxia).
2. Western blotting was used to measure protein levels of HIF-1α, the cytosolic subunit of HIF-1 that determines HIF-1 activation.

In this research work, we have demonstrated that under both 1% O_2 and 4% O_2, MnSOD suppressed the hypoxic induction of HIF-1. The results are as following:

I. HIF-1α protein is inducible in MCF-7 cells with hypoxia stimulation.

For this project, the proper cell line to work with should have inducible HIF-1α accumulation with hypoxia stimulation. That is, the cells have non-detectable or very low levels of HIF-1α protein at normal oxygen concentration (21% O_2) and have much higher expression level after hypoxia (1% O_2) treatment. To choose a proper cell line that has inducible HIF-1α protein, human breast adenocarcinoma MCF-7 cells were exposed to hypoxia (1% O_2) for 4 h. Then the protein was harvested and western blot was carried out on HIF-1α protein. **Figure 1** shows that MCF-7 cells have inducible HIF-1α protein under hypoxia. HIF-1α protein increased dramatically after 4 h treatment of hypoxia (1% O_2). Moreover, these cells have relatively lower MnSOD activity compared to other types of cancer cells. Therefore, it is a proper cell line we can use to test the effect of MnSOD activity elevation on hypoxic induction of HIF-1α protein.

II. MnSOD elevation by plasmid transfection blocks hypoxic accumulation of HIF-1α in MCF-7 cells exposed to 1% O_2.

To study the effects of MnSOD on HIF-1α protein expression, we first studied human breast carcinoma MCF-7 cells that had been stably transfected with plasmids containing MnSOD cDNA. These MCF-7 clones with different MnSOD activities have been characterized [Zhang HJ, Yan T, 1999]. We exposed these 13 different clones to 1% O_2 hypoxia for 4 h, and then analyzed HIF-1α protein levels with western blotting (**Figure 2**). Interestingly, we found that MnSOD suppressed hypoxic induction of HIF-1α. When MnSOD activity was moderately increased (2- to 6-fold relative to that of the parent cells), HIF-1α protein accumulation was suppressed. It decreased with increasing MnSOD activity until it was no longer detectable. However, when MnSOD activity increased to a higher level (> 6-fold), HIF-1α protein was again detected, and it increased with increasing MnSOD activity (**Figure 2a**). Regression analysis demonstrates a linear inverse correlation of HIF-1α with MnSOD activity at low levels of MnSOD (2- to 6- fold), whereas at high levels of MnSOD (> 6-fold), a linear positive correlation is observed (**Figure 2b**). In our previous studies we observed that when MnSOD activity was moderate (<6-fold), tumor growth was slowed [Zhang HJ, Yan T, 1999]. However, when MnSOD activity was increased beyond 6-fold, the tumor suppressive effect of MnSOD is diminished. This biphasic effect of MnSOD on tumor growth parallels the biphasic modulation of HIF-1α by MnSOD we observed here, suggesting that HIF-1α could be an effector of MnSOD mediated tumor suppression.

III. MnSOD elevation by adenoviral transduction blocks hypoxic accumulation of HIF-1α in MCF-7 cells exposed to 1% O_2.

In order to confirm and extend this observation using stable transfected cells, we further studied this effect in cells transduced with adenovirus expressing MnSOD. MCF-7 cells were transduced with different doses of adenoviral MnSOD. MnSOD enzymatic activity increased with increasing MOI (multiplicity of infection) of adenovirus, but CuZnSOD activity was unchanged (**Figure 6a**). Following adenovirus transduction, cells were exposed to 1% O_2 for 4 h. HIF-1α protein was then measured. As shown in **Figure 3**, adenovirus transduction of MnSOD suppressed HIF-1α protein levels under 1% O_2 similar to that with plasmid transfection. HIF-1α protein levels dropped significantly from 5 MOI of adenoviral MnSOD and became undetectable at 50 MOI; HIF-1α protein was again detectable at 75 MOI and increased with increasing MOI thereafter (**Figure 3a**), parallel to the effect observed with plasmid transfection of MnSOD. While the correlation is not as significant, the trend is similar (**Figure 3b**). In both plasmid transfections and adenovirus transduction experiments, HIF-1α protein was undetectable in cells grown under 21% O_2.

IV. MnSOD elevation blocks hypoxic accumulation of HIF-1α in MCF-7 cells exposed to 4% O_2.

MnSOD suppressed HIF-1α protein expression in MCF-7 cells exposed to 1% O_2, both with plasmid transfection and adenoviral transduction. We then studied if this suppressive effect also exists in cells exposed to 4% O_2. As shown in **Figure 4**, we used the same clones as those used under 1% O_2 (MCF-7 cells stably transfected with plasmids containing MnSOD cDNA), subjected them to 4% O_2, then studied HIF-1α protein expression. HIF-1α protein began to decrease in clones that expressed 2-fold MnSOD activity and remained suppressed in clones that had up to 6-fold MnSOD activity. However, HIF-1α levels were increased when MnSOD activity increased to 8-fold, though only a very small increase was observed and not in the dose dependent manner as seen under 1% O_2. **Figure 5** shows the effects on MCF-7 cells of adenoviral transduction of MnSOD. Once again, we found the suppressive effect similar to that observed with 1% O_2. HIF-1α was suppressed at a MOI of 50 and 75, then reappeared at a MOI of 100.

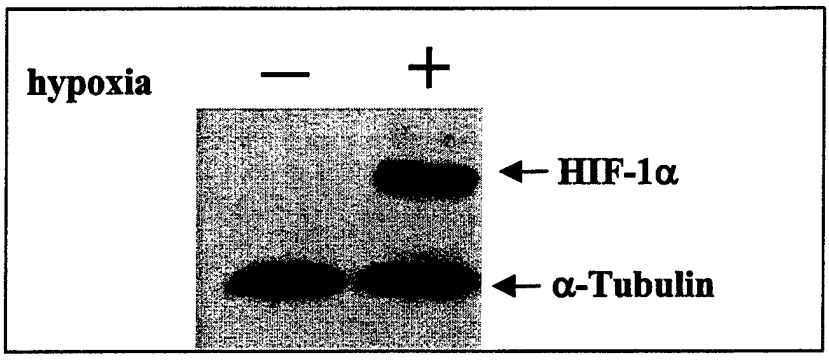


Figure 1. HIF-1α protein accumulated with hypoxic stimulation in MCF-7 cells. MCF-7 cells were exposed to either 21% O_2 or 1% O_2 for 4 h. Cells were then lysed and western blot was carried out to determine HIF-1α protein levels. α-tubulin was used as even loading control.

-: 21% O_2; +: 1% O_2.

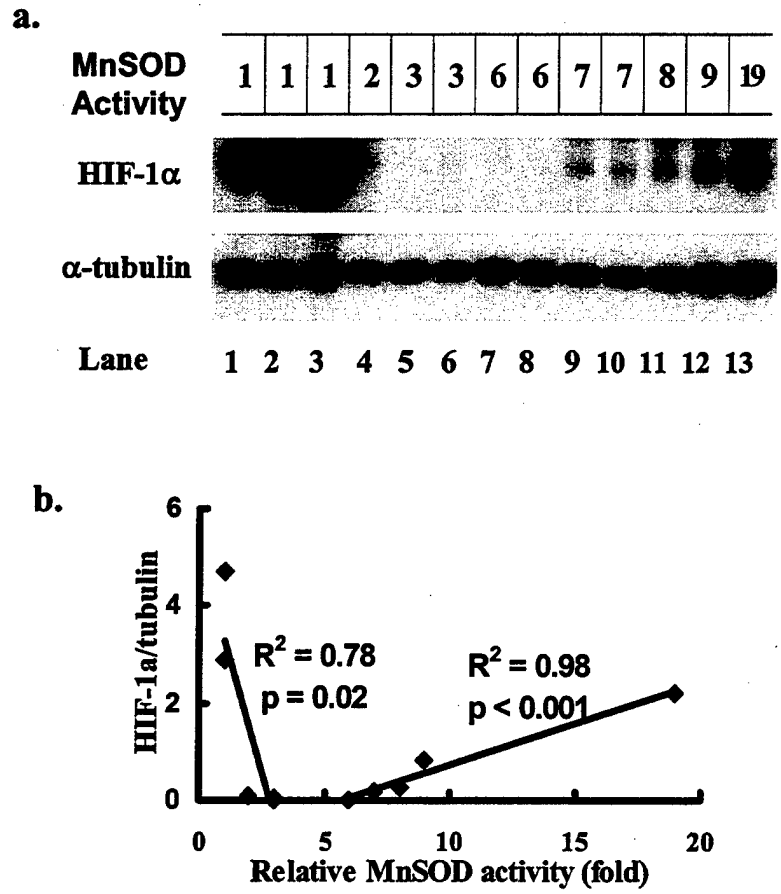


Figure 2. Plasmid transfection of MnSOD suppressed HIF-1α protein levels under 1% O_2.

a. Western blot showed HIF-1α protein expression in 13 different MnSOD stable transfection clones of MCF-7 cells after 4 h hypoxic stimulation (1% O_2). The first 3 lanes were samples from: MCF-7 parent cells, Neo vector control and clone with same activity as parent cells, respectively. The remaining 10 lanes were samples from clones with increasing MnSOD activity. The top row of numbers showed the relative MnSOD activity compared to the parent MCF-7 cells. α-tubulin was used as even loading control.

b. Regression analysis showed a biphasic effect of MnSOD on HIF-1α protein. HIF-1α protein levels were quantified by densitometry and normalized to α-tubulin levels. Similar results have been obtained in repeated experiments.

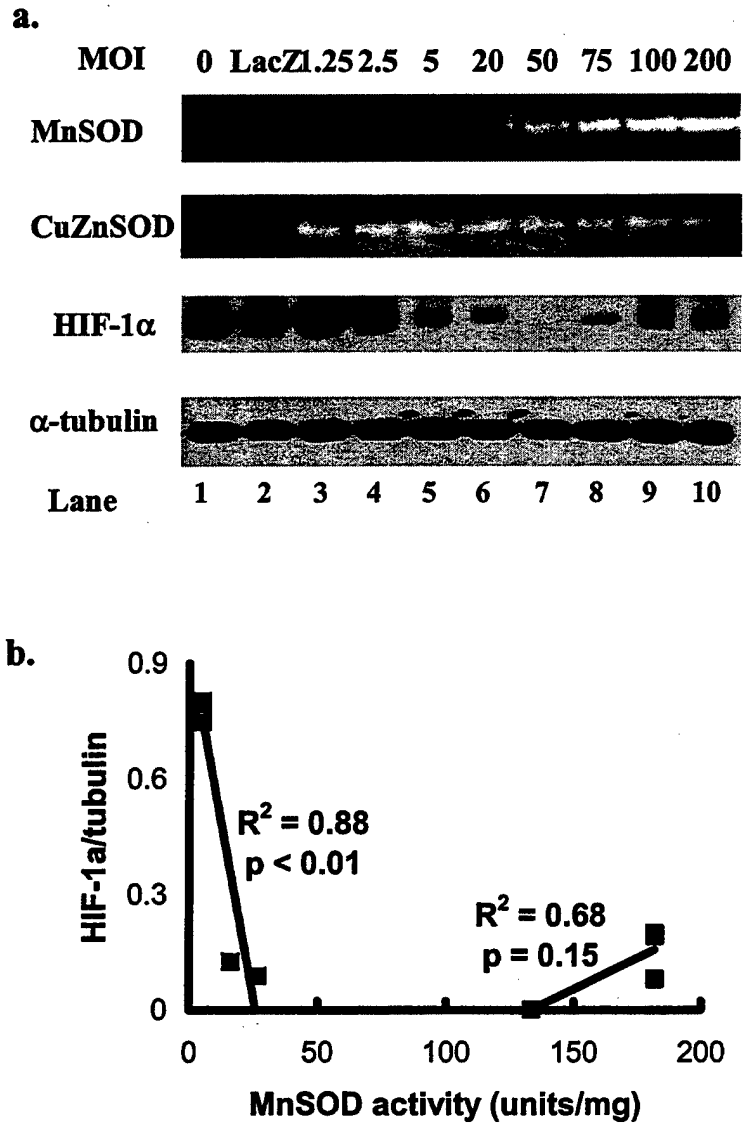


Figure 3. Adenoviral transduction of MnSOD suppressed HIF-1α protein levels under 1% O_2.

a. Effect of adenoviral transduction of MCF-7 cells. The top two panels showed that MnSOD activity increases with increasing MOI (from 1.25 up to 200), while CuZnSOD activity did not change. The third and fourth panels showed western blots for HIF-1α and α-tubulin, respectively.

b. Regression analysis showed a biphasic effect of MnSOD on HIF-1α protein. HIF-1α protein levels were quantified by densitometry and normalized to α-tubulin levels. Similar results have been obtained in repeated experiments.

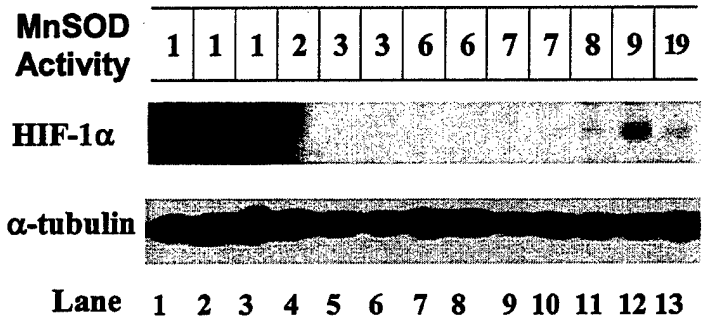
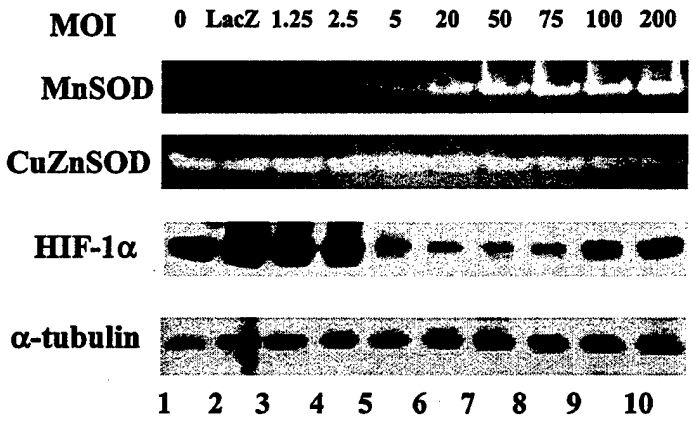


Figure 4. Plasmid transfection of MnSOD suppressed HIF-1α protein levels under 4% O_2. Western blot showed HIF-1α protein expression in different MnSOD stable transfection clones of MCF-7 cells under 4% O_2. The 13 different clones were the same as used in **Figure 5**. The top row of numbers showed the relative MnSOD activity compared to the parent MCF-7 cells. Similar results have been obtained in repeated experiments.

a.



b.

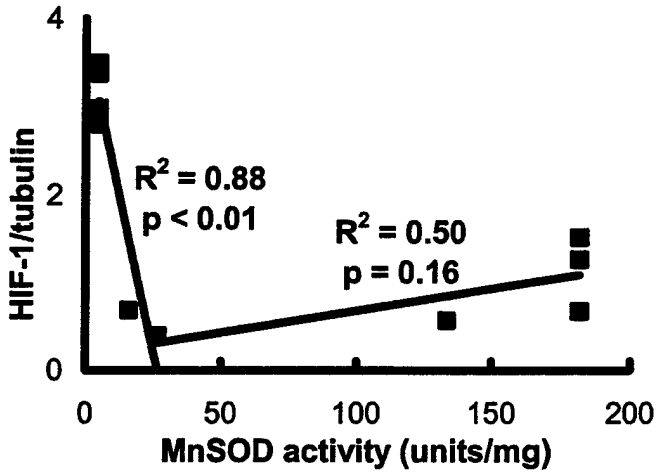


Figure 5. Adenoviral transduction of MnSOD suppressed HIF-1α protein levels under 4% O_2.

a. Effect of adenoviral transduction of MCF-7 cells. The top two panels showed that MnSOD activity increased with increasing MOI (from 1.25 up to 200), while CuZnSOD activity did not change. The third and fourth panels showed western blots for HIF-1α and α-tubulin, respectively.

b. Regression analysis showed a biphasic effect of MnSOD on HIF-1α protein. HIF-1α protein levels were quantified by densitometry and normalized to α-tubulin levels. Similar results have been obtained in repeated experiments.

Key Research Accomplishments

The key research accomplishments are the results we have demonstrated above:

I. We have clearly demonstrated that MnSOD suppressed hypoxic induction of HIF-1, which is the overall objective of this research work.

II. We have obtained the human breast carcinoma MCF-7 cells that have been stably transfected with MnSOD and vector controls.

III. Human breast carcinoma MCF-7 cells were also transduced with adenoviral containing human MnSOD cDNA.

IV. A proper condition has been established to induce HIF-1 expression in these cells.

V. Western blotting was used to measure protein levels of HIF-1α, the cytosolic subunit of HIF-1 that determines HIF-1 activation.

Reportable Outcomes

The following abstracts have resulted from this research work:

1. **"Modulating HIF: Potential Targets for Cancer Therapy".** M. Wang, H.J. Zhang, J.S. Kirk, L.W. Oberley, G.R. Buettner. 2nd International Coference on Prostate Cancer Research, 2002.

2. **"MnSOD suppresses hypoxic induction of HIF-1".** M. Wang, H.J. Zhang, J.S. Kirk, L.W. Oberley, and G.R. Buettner, 9th Annual Meeting of the Oxygen Society, 2001.

3. **"The Connection Between HIF-1 and SODs: Potential Targets for Cancer Therapy".** M. Wang, H. Zhang, J. Kirk, Y. Zhang, F.Q. Schafer, L.W. Oberley, G.R. Buettner. The University of Iowa Research Week, 2001.

4. Half way to my Ph.D. thesis.

Conclusions

Due to inefficient vascular supply and the resultant reduction in tissue oxygen tension, hypoxia is widespread in solid tumors. Hypoxia is a stimulus that can lead to neovascularization in order to satisfy the needs of the tumor [Liu Y *et al.*, 1995]. Hypoxia-inducible factor-1 (HIF-1) is an important transcription factor that is activated in conditions of decreased oxygen [Wang GL & Semenza GL, 1993a]. It mediates cell survival in hypoxia by promoting genes involved in glucose homeostasis, erythropoiesis, and angiogenesis. While normal tissue and benign breast tumors do not exhibit increased HIF-1 activity, evidence for a graded increase in activity has been demonstrated in the progression from pre-neoplastic lesions to cancer metastases [Bos R *et al.*, 2001]. Moreover, a positive correlation has been found between HIF-1 expression and vascularization in tumors [Zagzag D, Zhong H, 2000].

MnSOD is an important antioxidant enzyme involved in cancer cell growth. It has been shown both *in vitro* and *in vivo* that tumor growth is suppressed with MnSOD overexpression. It has been proposed that MnSOD is a tumor suppressor gene [Oberley LW & Buettner GR, 1979; Bravard A *et al.*, 1992].

We compared the HIF-1α protein level with hypoxia stimulation in MnSOD overexpressing cells. Our results demonstrated that MnSOD surprisingly modulated hypoxic induction of HIF-1α. Moderate levels of MnSOD activity (2- to 6-fold compared to parent cells) in human breast carcinoma MCF-7 cells abolished the accumulation of HIF-1α protein at both 1% and 4% O_2. However, higher level of MnSOD allowed the accumulation, showing a biphasic effect. Interestingly, HIF-1 never returns to values near untreated cells; in other words, HIF-1α is still suppressed relative to parent cells and vector control. These results are consistent with what we have observed with tumor growth after MnSOD overexpression. That is, the rate of tumor growth slowed down when MnSOD activity was increased to a moderate level (<6-fold). However, MnSOD could not suppress the growth further when the activity increased to a higher level (>6-fold), and indeed some of the tumor suppression effect was lost [Zhang HJ, Yan T, 1999]. This suggests that the inhibition of HIF-1 accumulation may account for the tumor suppressor function of MnSOD enzyme. By controlling HIF-1 protein and thereby controlling the expression of HIF-1 downstream genes that are involved in tumor metabolism and growth, MnSOD control the switch for the genes that allow tumor progression.

This is the first time that a direct connection between HIF-1 alpha and SOD has been shown. These results indicate that SOD regulates HIF-1 alpha and the modulation of HIF-1 alpha protein levels may account for the tumor suppressor function of SOD enzymes.

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